UCC_Ireland_SeeDNA_Subproject_Protocols

Much of our work involved theoretical design of the detector plasmid. This included bioinformatics, such as sequence analysing, primer design, target design and choosing restriction enzymes. Hence, only a handful of simple protocols were carried out over the course of the experiment to construct and assess our detector. Exact details of the construction are not described below to allow the option of patenting our process and design, if future results indicate this potential.

1. Making Agarose Gels

We decided to make 1% agarose gels for the duration of our project as they separate DNA whose sizes are between 250 base pairs to 12 Kb. This is optimal for our working range. This required us to make 5X TBE which was prepared as follows:

• 54g Tris Base

• 27.5g Boric Acid

• 3.7 grams EDTA

This was then dissolved in 800mL of DH2O and then made up to one litre by adding water. It was then diluted 5 fold to 1X TBE working solution when required.

This buffer was used to dissolve agarose. A 1% gel was made by dissolving 0.5g agarose in 50ml 1X TBE and pouring into a mould to solidify. A safeview stain was used to visualize the DNA under U.V light. This was added prior to pouring. 5µL was used for a 50mL. 6x purple loading dye, provided in the iGEM kit from NEB, was used when loading most DNA samples.

2. Plasmid Digests

A number of digests were carried out throughout these experiments. Many of these were a central part to the construction of our detector plasmid and hence, at this time, cannot be fully discussed. However digests were carried out using NEB enzymes with appropriate buffers. Digests were generally confirmed by running a sample of the digest on an agarose gel and examining bands produced.

3. Gel Extractions

QIAquick Gel Extraction Kit protocol was used to extract digested sequences from agarose gels. The removed constructs were then used in further experiments. The protocol was as follows:

1. The DNA fragment was excised from the gel using a clean scalpel and forceps, and placed into a sterile eppendorf tube.
2. The gel slice was weighed by calculating the difference between the empty and gel-containing tube.
3. 3 volumes of Buffer QG were added to one volume of gel (eg. 100mg gel in 300ul buffer)
4. This was incubated at 50°C for 10 min, and vortexed at intervals to ensure complete dissolution.

5. 1 Gel volume of isopropanol was added to the sample and mixed/
6. A QIAquick spin column was placed in a 2ml collection tbe
7. The sample was applied to the spin column and centrifuged at max speed for 1 min
8. The flow-through was discarded and the spin column replaced to the collection tube.
9. 0.5ml Buffer QG was then added to the column and spun for 1min to remove traces of agarose.
10. The sample was washed by adding 0.75ml buffer PE to the column and centrifuging for 1 min.
11. The flow through was discarded and the column spun again to remove excess buffer.
12. The spin column was then transferred to a new collection rtube.
13. 0.05ml elution buffer was applied to the column and allowed to incubate at room

4. Mini Preps

This was carried out at multiple steps in the experiment. Preps were needed to isolate the original plasmid used. Also, preps were carried out on coloniers, post detection of a target, to send the plasmid for sequencing to confirm results. The GenElute Plasmid mini prep kit was used, the protocol is as follows:

1. Use 5ml of the overnight culture.
2. Pellet cells at maximum speed for 1 minute, discard supernatant.
3. Resuspend pellets in 200 µL of resuspension solution, pipette up and down well to resuspend.
4. Lyse cells, add 200 µL of lysis reagent, invert gently 5-6 times.
5. Immediately, add 350 µL of neutralisation solution, invert gently 5-6 times.
6. Centrifuge at top speed for 10 minutes. If there are floaty white bits, spin again.
7. Apply clear lysate to spin column.
8. Centrifuge at top speed for 1 minute, discard flow through.
9. Add 750 µL of wash solution, centrifuge at top speed for 1 minute, and discard flow through.
10. Centrifuge empty column for 1 minute more at maximum speed.
11. Transfer column to a fresh collection tube.
12. Add 100 µL of elution buffer/sterile water, incubate for 1 minute.
13. Centrifuge at top speed for 1 minute.
14. Measure DNA concentration.

5. Transformation into Competent DH5a Cells

Protocol used:

1. Incubate agar plates with chloromphenicol at 37°C, use one plate for control.
2. Use 100µL of DH5α cells for each transformation, make sure everything is on ice.
3. Add 1-5µL of vector to cells and mix gently
4. Incubate on ice for 25 minutes.
5. Heat shock at 42°C for 1 minute maximum.
6. Put cells back on ice for 5 minutes.
7. Add 1ml of LB broth as a medium.
8. Incubate shaking at 37°C for 1 hour.
9. Plate up cells- Spin cells at 3k for 3 minutes, quickly pour off supernatant, leaving 100µL of broth and suspend cells.

10. After plating up cells, incubate plates at 37°C overnight and check for colonies the next morning.

6. Phosphorylation of Oligos

This was carried out on primers and target sequences commercially synthesised to allow ligation and annealing to our DNA samples. This reaction was carried out as:

1. Dehydrated oligos were resuspended in nuclease-free water to give a 100mM stock
2. Reaction was set up as:
   - 100 µM oligo stock 2 µL
   - 10X T4 DNA ligase buffer 2 µL
   - Sterile water 15 µL
   - T4 Polynucleotide Kinase 1 µL
3. This was allowed to incubate at 37^0C for 1 hour T4 PNK was then heat inactivated at 65^0C for 20 minutes.
4. The phosphorylated oligos were then stored at -20^oC

7. Annealing of HPV16 phosphorylated strands

20ul of the top strand phosphorylated oligonucleotide was mixed with 20ul of the bottom strand nucleotide. The mixture was heated for 5mins at 95°C in a PCR machine heat block. The PCR machine was programmed to cool the mixture to room temperature over 90mins. The two single-stranded DNA molecules annealed to each other, forming the HPV16 target sequence. It was then stored at -20°C.

8. Cloning of the HPV and Sry Detector into PSBC13 Plasmid

1. Detector inserts were ordered as a double stranded oligos, along with flanking biobrick prefix and suffix sequences.
2. Oligo was digested with EcoR1 and Pst1 restriction enzymes, located on the biobrick prefix and suffix sequences.
3. Plasmids pSBC13 and pSBC13 with Biobrick Part Number K584001 (constittive GFP) were each digested with restriction enzymes EcoR1 and Pst1
4. Oligo was then ligated into the plasmids.
5. Plasmids were transformed into DH5- α cells and grown overnight on separate plates.
6. Colony PCR was carried out on colonies from each plate, along with two from control plates containing control colonies. The primers used were VF2 and a reverse detector primer.
7. A number of colonies were also selected for sequencing to confirm presence of the insert.

9. Cloning of the HPV and Sry Targets into pBluescript Plasmid

1. Target inserts were ordered as a overlapping oligos, along with flanking XhoI and EcoRI restrictions site recognition sequences.

2. A primer extension reaction was performed using Phusion Hotstart DNA polymerase (Thermo Fisher).
3. Product was digested with EcoR1 and XhoI restriction enzymes.
4. Plasmid pBluescript SK II + (Agilent Technologies) was digested with same restriction enzymes
5. Product was then ligated into the plasmid.
6. Plasmids were transformed into DH5- α cells and grown overnight on separate plates.
7. A number of colonies were also selected for sequencing to confirm presence of the insert.

10. Detection reaction

1. A typical detection reaction involved mixing approximately 10ng of detector plasmid with 0.1 picomoles of oligonucleotide target or 10ng of HaeIII digested target plasmid.
2. Further details of the detection reaction are not provided for commercial reasons.
3. The detection reaction was followed by transfromation into E. coli as outlined above.